

Caspase Cleavage Is Not for Everyone

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During apoptosis, caspases cleave cellular substrates to break down and package the apoptotic cell for removal. Reporting in *Cell*, Mahrus et al. (2008) and Dix et al. (2008) use new approaches that identify hundreds of previously unrecognized caspase substrates, many of which appear to produce polypeptide fragments with potentially new functional activities.

Caspases (cysteine-dependent aspartate-directed proteases) are the chief executioners of apoptotic cell death, transforming intact cells into parceled remnants suitable for phagocytic ingestion. Although dismantling of cells by caspases could, in theory, result from indiscriminate proteolysis, the packaging of apoptotic cells might also reflect the selective proteolysis of precise cellular substrates. Distinguishing between these possibilities requires a comprehensive identification and functional analysis of caspase substrates. Many caspase substrates have been identified either through large-scale *in vitro* cleavage of peptide libraries, which provides no information about physiological relevance, or through analysis of individual proteins by gel electrophoresis, which is low throughput and often relies on the availability of specific antibodies to track proteolytic fragments. Two new studies in *Cell* (Dix et al., 2008; Mahrus et al., 2008) address deficiencies in these approaches by developing proteomics-based platforms that enable large-scale profiling of proteolytic events.

The two groups each use unique methodologies to analyze caspase-mediated proteolysis in apoptotic Jurkat T cells. Dix et al. (2008) developed PROTOMAP, a platform that combines gel electrophoresis (SDS-PAGE) with mass spectrometry (shotgun LC-MS/MS) to visually compare the size, topography, and abundance of proteins in control cells versus cells treated with staurosporine, a kinase inhibitor that induces apoptosis. This methodology offers the unprecedented ability to detect all cleavage fragments without access to specific immunological reagents. Mahrus et al. (2008) present a technology that

captures N-terminal peptides produced during etoposide-induced apoptosis by cleverly taking advantage of biotinylation of α -amines (and not lysine ϵ -amines) by subtiligase. This approach allows selective N-terminal labeling (including *de novo* N termini produced by caspase cleavage), capture of labeled peptides on an avidin resin, and LC-MS/MS analysis to determine the precise sites of proteolytic cleavage. Such strategies for comprehensive investigation of proteolytic events provide an important precedent for the analysis of other types of proteolysis and of caspase-dependent proteolysis induced by different stimuli or in different cell types.

Despite their different methodologies, both studies uncovered hundreds of previously unreported caspase substrates. With Dix et al. (2008) documenting 170 new substrates and Mahrus et al. (2008) finding 240 new substrates, these data highlight our incomplete knowledge of caspase-mediated proteolysis. Indeed, Lüthi and Martin (2007) recently compiled every caspase substrate published to date in a searchable database (CASBAH), with substrate numbers upwards of 400. Given that the lists generated by CASBAH, Dix et al., and Mahrus et al. only have minimal overlap (Figure 1A), it is likely that the list of substrates will only continue to grow. Interestingly, quantitation of cleavage events in the Dix et al. report suggest that previous studies may have been limited by low sensitivity in that only the more abundant substrates in the PROTOMAP data set had been shown previously to be cleaved during apoptosis.

Given the vast number of substrates, are caspases acting indiscriminately, or is there some method to the madness?

Certain substrates are known to be crucial for affecting typical apoptotic morphology, such as membrane blebbing induced by cleavage of the Rho-associated kinase ROCK1 and DNA fragmentation induced by cleavage of the endonuclease CAD/DFF40 (Coleman et al., 2001; Woo et al., 2004). But are these examples the exception or the rule? Intriguing data from each study suggest that an active caspase is not a haphazard “Pac-Man” but rather a purposeful and incisive surgeon. Perhaps most surprising is the observation by Mahrus et al. that caspases appear to target multiple proteins within single protein complexes or biochemical pathways. Their analysis most strongly implicates proteins involved in the regulation of transcription as caspase targets but also identifies subnetworks of substrates in 11 other pathways, including those that regulate DNA repair and block apoptosis. These data suggest that caspases may coordinately control specific biochemical pathways or signaling networks.

A second surprising result, reported by Dix et al., suggests that more than one-third of proteolytic fragments generated by caspase cleavage are stable for at least 4 hr (in cells that died within 6 hr after apoptotic stimulation). This is in contrast to the notion that cleaved caspase substrates are rapidly degraded. For example, Ditzel et al. (2003) reported that caspase-mediated cleavage of DIAP1, a *Drosophila* inhibitor of apoptosis protein (IAP), generates an unstable N-degron that quickly undergoes complete degradation via the N-end rule pathway. Although the global analysis of caspase substrates suggests that production of stable fragments may be frequent, caspase cleavage-induced deg-

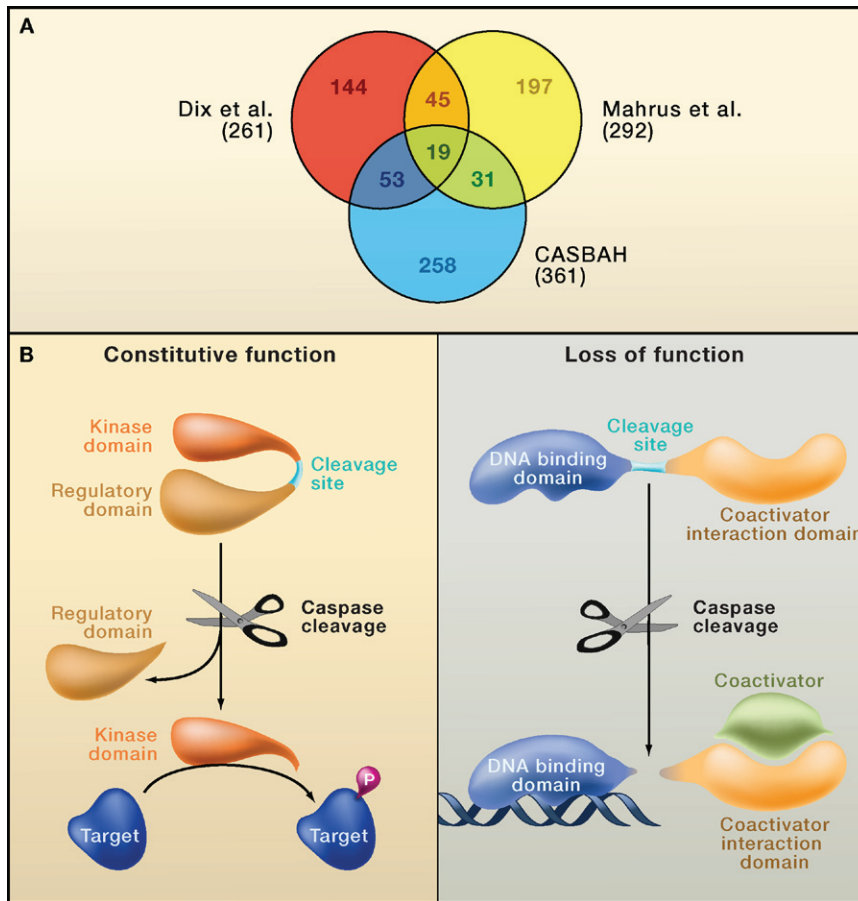


Figure 1. Caspase Substrates and Potential Functions of their Cleavage Products

(A) Depicted is the overlap between the caspase substrates in apoptotic Jurkat T cells identified by Dix et al. (2008) and Mahrus et al. (2008) and the previously reported human caspase substrates as compiled in the CASBAH database (Lüthi and Martin, 2007). The total number of substrates reported in each study is indicated in parentheses.

(B) Caspase-mediated cleavage of a cellular substrate resulting in separation of functional domains could lead to either constitutive activity of one of the proteolytic fragments (left) or to loss of function of the substrate (right). Depicted is the separation of a kinase domain from its regulatory domain, which allows for unchecked phosphorylation of target proteins (left). Conversely, separation of a DNA binding domain from a coactivator interaction domain within a transcription factor would prevent coactivator recruitment to the DNA, thereby hindering transcriptional induction (right).

radation of antiapoptotic proteins (such as DIAP1) makes sense as a means for “releasing the brakes” on apoptosis. Future mining of the PROTOMAP data might query whether cleavage of other antiapoptotic substrates produces unstable fragments.

Even more interesting, Dix et al. noticed that a number of the persistent peptide fragments mapped to distinct, stably folded protein domains, leading them to speculate that caspase-mediated proteolysis yields a class of effector protein fragments with new activities. Mahrus et al. were similarly interested in whether caspases might preferentially

cleave substrates between functional domains, but their analysis demonstrated an even split of sites within or between domains. That said, data from both groups support the concept that a substantial portion of caspase substrates are cleaved into domain-containing fragments. As to the purpose of such cleavage events, Mahrus et al. speculate that physical separation of functional domains can lead to protein inactivation (or perhaps production of dominant-negative fragments). Given that Dix et al. predict cleavage-induced activation of domain-containing fragments, how does one resolve this apparent discrepancy?

Perhaps the simple answer is that both models are correct, depending on the individual substrate in question (Figure 1B). It has long been appreciated that cleavage of kinases can release constitutively active domains (such as cleaved HPK1, a modulator of the JNK pathway), whereas cleavage of other substrates, such as poly (ADP-ribose) polymerase (PARP), is associated with loss of function (Chen et al., 1999; D’Amours et al., 2001).

The techniques described in these new reports provide powerful methods for global analysis of caspase-mediated proteolysis. Collectively, the data suggest that dismantling the apoptotic cell is more akin to folding a tent after careful removal of the pegs than to gathering and disposing of indiscriminately destroyed debris after an explosion. Caspases selectively target certain biochemical pathways, generating a unique set of stable protein fragments possessing new functionality within the dying cell. Although an impressive start, the work leaves much left to do. Given the large number of substrates identified, one of the most daunting but important next steps will be to verify each putative caspase substrate and determine what, if any, functional significance it may have in mediating the execution of apoptosis.

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